LOCALIZATION OF A 34 000 AND A 23 000 M_r POLYPEPTIDE TO THE LUMENAL SIDE OF THE THYLAKOID MEMBRANE

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1. Introduction

Several attempts have been made to localize different components across the thylakoid membrane. The methods used include treatments with membrane-impermeable agents such as antibodies [1], chemical modifiers [2,3] and proteolytic enzymes [3]. This has given valuable information about the outer surface of the thylakoid membrane and has revealed that components like ferredoxin, ferredoxin–NADP-reductase, the coupling factor and the light-harvesting complex are all accessible from the outside. Information about the inner thylakoid surface is poor however and the localization of components on this side has often been deduced from indirect measurements. A direct study of the inner side has become possible after the isolation of inside-out thylakoids [4–9].

In [10] trypsination of inside-out thylakoid vesicles was used to demonstrate that at least a part of the water-splitting system is exposed to the lumen.

Here, inside-out thylakoid vesicles have been treated with trypsin or alkaline-Tris. The subsequent changes in the polypeptide pattern were followed by SDS–PAGE.

2. Materials and methods

2.1. *Preparation of thylakoid membrane fractions*

Stacked spinach thylakoid membranes [11] were fragmented by a Yeda press disintegration procedure [4]. Inside-out thylakoid vesicles formed during this procedure were separated from right-side-out material by partition in an aqueous dextran–polyethylene glycol two-phase system [4]. Chloroplast fragments were added to a polymer mixture to yield the following composition: 5.7% (w/w) Dextran T-500, 5.7% (w/w) polyethylene glycol 4000, 10 mmol/kg sodium phosphate buffer (pH 7.4), 5 mmol/kg NaCl, 20 mmol/kg sucrose and chloroplast material corresponding to 160–240 μg chl/ml. The phase system was carefully mixed and allowed to settle. The inside-out material, partitioning to the lower phase, was purified by repeating the partition procedure twice with pure upper phase. The purified inside-out thylakoids were removed from the polymers by dilution and centrifugation for 30 min at 100 000 × *g* and finally resuspended in 10 mM sodium phosphate buffer (pH 7.4) containing 5 mM NaCl and 100 mM sucrose. Destacked thylakoids were used as the right-side-out material.

2.2. *Trypsin treatment*

Trypsin (Sigma type XI) treatment was at 20°C with a sample concentration corresponding to 400 μg chl/ml. The digestion was stopped after 2.5 min by injection of solubilizing buffer (see below) at 80°C and kept at 80°C for 2 min.

2.3. *Tris treatment*

Tris treatment was carried out by incubating the membranes at a concentration corresponding to 200 μg chl/ml, in 0.8 M Tris (pH 8.0) for 30 min and 4°C. The incubation was followed by centrifugation for 30 min at 100 000 × *g* for inside-out thylakoids and 10 min at 2000 × *g* for right-side-out thylakoids.

Abbreviations: chl, chlorophyll; Tris, tris(hydroxymethyl) aminoethane; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; M_r, relative molecular mass

The sediments and supernatants were collected and dialyzed against 0.1% SDS in order to remove remaining Tris. Before solubilization the sediments were set to 400 μg chl/ml while the supernatants were enriched to give a staining comparable to the sediments.

2.4. *SDS–polyacrylamide gel electrophoresis*

Samples were solubilized for 2 min at 80°C in a medium of the following composition: 5% (v/v) mercaptoethanol, 2% (w/v) SDS, 150 mM sucrose, 6 mM EDTA and 54 mM Tris–SO_4 buffer (pH 6.10). Electrophoresis was performed in the discontinous buffer system [12] using slab gels with an acrylamide gradient of 13–17% (2.7% crosslinking). Electrophoresis was run for 6 h at 20°C and a constant current density of 10 mA/cm^2. The gels were stained with Coomassie brilliant blue R250. The M_r standards were bovine serum albumin (68 000), ovalbumin (43 000), soybean trypsin inhibitor (21 600) and myoglobin (17 200).

3. Results

3.1. *Trypsin treatment*

Several membrane polypeptides were degraded by trypsin in both right-side-out and inside-out thylakoid fractions (fig.1). Of special interest is a 34 000 M_r polypeptide which was degraded exclusively in the inside-out fraction, demonstrating that this polypeptide is exposed at the lumenal side of the thylakoid membrane. The polypeptide pattern for treated right-side-out thylakoids reveals that polypeptides with app. M_r 25 000 and 26 000–27 000, probably representing apoproteins of the light-harvesting complex, were almost quantitatively degraded. These results for right-side-out thylakoid membranes are in good agreement with [13,14]. These polypeptides were also slightly affected in the inside-out fraction. This effect varied between different preparations and may to some extent be due to contamination by right-side-out material in the inside-out fraction or to limited penetration of the enzyme.

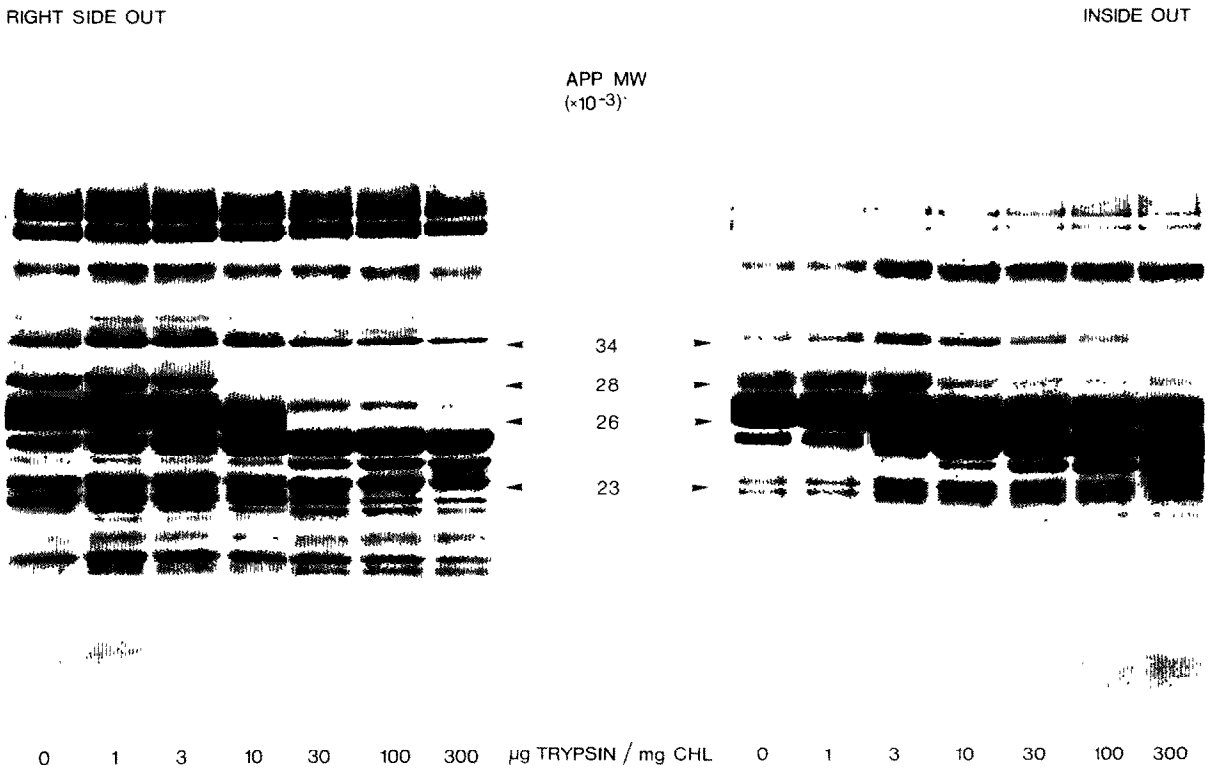

Fig.1. Effects of trypsin on the polypeptide pattern for thylakoids of opposite sidedness.

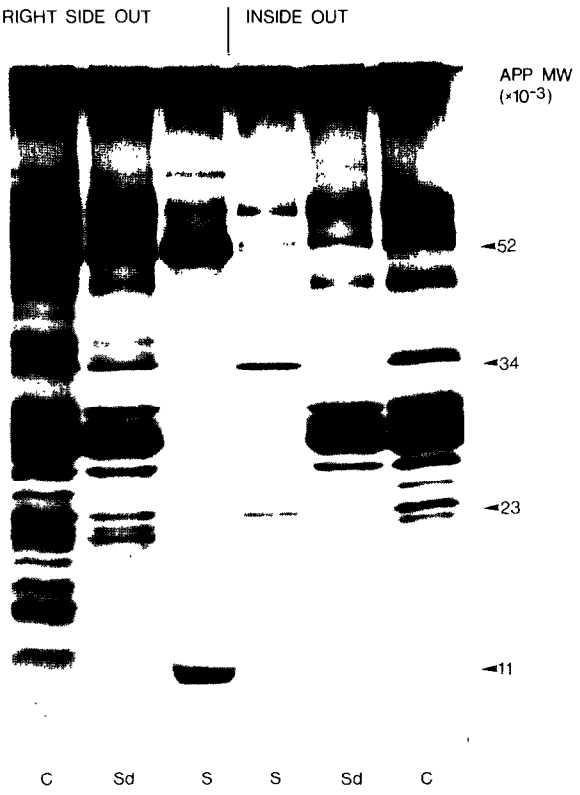

Fig.2. Effects of Tris washing on the polypeptide pattern for thylakoids of opposite sidedness: C, control; Sd, sediment after Tris washing; S, supernatant after Tris washing.

3.2. *Tris treatment*

Alkaline Tris washing removed several polypeptides from the thylakoids (fig.2). From the right-side-out material there were only two polypeptides that were quantitatively removed. One in the 52 000–53 000 M_r region represents the large subunit of ribulose 1,5-bisphosphate carboxylase (or possibly the β-subunit of the coupling factor). The other, with app. M_r 11 000, is presumably the small subunit of the carboxylase.

For the inside-out fraction, two polypeptides with app. M_r 34 000 and 23 000 were completely removed from the thylakoids by Tris washing and recovered in the supernatant. In contrast to the right-side-out material, these polypeptides were found in the sediment fraction.

4. Discussion

The trypsin and Tris treatments demonstrated independently that a 34 000 M_r polypeptide was exposed to the surrounding medium in the inside-out but not in the right-side-out thylakoid fraction. Furthermore, the Tris treatment also removed a 23 000 M_r polypeptide specifically from the inside-out thylakoids. These two polypeptides are therefore suggested to be extrinsic and located at the inner surface of the native thylakoid membrane.

Several observations suggest that the 34 000 M_r polypeptide is a part of photosystem II. In [15] a 34 000 M_r polypeptide was enriched in photosystem II fractions but almost completely absent in photosystem I fractions. In [16,17] mutants, of *Scenedesmus* and *Zea* maize, respectively, showed a total or partial loss of a polypeptide in the 32 000–34 000 M_r region. The loss of this polypeptide was correlated with a loss of water-splitting capacity [16] while the reaction centre activity of photosystem II seemed to be unaffected [16,17]. In [18] a 33 000 M_r polypeptide has been isolated, characterized and ascribed a function in photosystem II.

In [10], mild trypsination, under conditions similar to those described here, severely inhibited the water splitting activity in inside-out thylakoids, while the right-side-out thylakoids were only slightly affected. The clear effect of trypsin on the 34 000 M_r polypeptide specifically in inside-out thylakoids (fig.1) and the ascribed function of this polypeptide in the water-splitting system [16] suggest that the trypsin effect on the water-splitting reaction in inside-out thylakoids [10] is due to the degradation of this 34 000 M_r polypeptide.

It is interesting to note that treatments with as high as 2 M NaCl quantitatively release the 23 000 M_r but not the 34 000 M_r polypeptide from the inside-out thylakoids [19]. This suggests that the release of the 34 000 M_r polypeptide upon Tris treatment has a more specific cause than just charge shielding. One known effect of alkaline-Tris is the extraction of Mn^{2+} [20,21]. If this implies that Mn^{2+} is involved in the binding of the 34 000 M_r polypeptide to the membrane or if the release of the polypeptide and Mn^{2+} are independent effects of Tris treatment is an open question.

In conclusion, two polypeptides with app. M_r 34 000 and 23 000 have been found to be exposed on the lumenal side of the thylakoid membrane. Furthermore, the 34 000 M_r polypeptide is probably associated with the water-splitting complex.

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